

Thermal Gradient Gel Electrophoresis Analysis of Bioprotection from Pollutant Shocks in the Activated Sludge Microbial Community

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We used a culture-independent approach, namely, thermal gradient gel electrophoresis (TGGE) analysis of ribosomal sequences amplified directly from community DNA, to determine changes in the structure of the microbial community following phenol shocks in the highly complex activated sludge ecosystem. Parallel experimental model sewage plants were given shock loads of chlorinated and methylated phenols and simultaneously were inoculated (i) with a genetically engineered microorganism (GEM) able to degrade the added substituted phenols or (ii) with the nonengineered parental strain. The sludge community DNA was extracted, and 16S rDNA was amplified and analyzed by TGGE. To allow quantitative analysis of TGGE banding patterns, they were normalized to an external standard. The samples were then compared with each other for similarity by using the coefficient of Dice. The Shannon index of diversity, H , was calculated for each sludge sample, which made it possible to determine changes in community diversity. We observed a breakdown in community structure following shock loads of phenols by a decrease in the Shannon index of diversity from 1.13 to 0.22 in the noninoculated system. Inoculation with the GEM (*Pseudomonas* sp. strain B13 SN45RE) effectively protected the microbial community, as indicated by the maintenance of a high diversity throughout the shock load experiment (H decreased from 1.03 to only 0.82). Inoculation with the nonengineered parental strain, *Pseudomonas* sp. strain B13, did not protect the microbial community from being severely disturbed; H decreased from 1.22 to 0.46 for a 3-chlorophenol–4-methylphenol shock and from 1.03 to 0.70 for a 4-chlorophenol–4-methylphenol shock. The catabolic trait present in the GEM allowed for bioprotection of the activated sludge community from breakdown caused by toxic shock loading. In-depth TGGE analysis with similarity and diversity algorithms proved to be a very sensitive tool to monitor changes in the structure of the activated sludge microbial community, ranging from subtle shifts during adaptation to laboratory conditions to complete collapse following pollutant shocks.

Shock loads of pollutants represent a significant hazard in wastewater treatment systems because they disturb the microbial community, resulting in loss of mineralization activity. To restore activity, time-consuming and costly measures must be taken. Therefore, as far as possible, shock loads are prevented from entering the plants by buffering tanks (1). Alternately, specialized inocula could be kept ready to protect the activated sludge microbial community from pollutant shock loads and thus allow continued functioning of the plant. However, to select strains which are appropriate for the pollutants in question and to ensure their effectiveness in bioprotection, an in-depth analysis of the community response to the shock, with and without inoculation with specialist inocula, is required.

Analysis of cloned ribosomal gene sequences directly retrieved from nature is the state-of-the-art technique for determining microbial community structure without bias introduced by cultivation (10). However, the high sample throughput required to determine community responses to experimental treatments cannot be achieved by the time-consuming analysis of clone libraries, in spite of significant improvements in sequencing and cloning techniques. As one attempt to obtain an overview of the structural diversity of microbial communities, denaturing gradient gel electrophoresis analysis (TGGE/DGGE) has been introduced into microbial ecology (16). It is based on the separation of ribosomal gene sequences directly amplified

from community DNA by using conserved primers on a denaturing gel according to their melting point.

In comparatively simple microbial communities, e.g., hot spring microbial mats or enrichment cultures, individual TGGE/DGGE bands can be assigned to cultured organisms or retrieved ribosomal sequences (8, 9, 24, 29). This is usually not possible in activated sludge, sediments, soil, and other highly diverse microbial systems because the banding patterns are much too complex. However, the number, precise position, and intensity of the bands reflect the number and relative abundance of dominant rDNA types in the sample and thus allow a comparison of microbial communities with each other. To be able to perform such analyses, we normalized the TGGE gels with respect to the reference standards included in all the gels. We then calculated the Dice coefficient of similarity between banding patterns of different gel strips. This allowed us to generate dendrograms and thus to group the samples according to the similarity of their community profiles. As a measure of the structural diversity of the microbial community, we calculated the Shannon index of general diversity (26) from the number and relative intensities of bands on an individual gel strip. We thus obtained a distinct diversity value for each sample and were able to observe changes in community diversity over time in different experiments.

We analyzed the efficiency of a genetically engineered microorganism (GEM) relative to its parental strain (*Pseudomonas* sp. strain B13) in protecting the activated sludge microbial community from self destruction through unproductive catabolism of methylated and chlorinated phenols. Microorganisms degrade these compounds via *meta*- and *ortho*-cleavage degra-

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dation pathways, both of which are generally present in bacteria, although usually only one type is functional, depending on the substrate available (20). However, if microbial communities are confronted with mixtures of substituted aromatics, both *meta*- and *ortho*-cleavage degradation pathways induce, resulting in metabolic chaos, i.e., misrouting of substituted catechols and accumulation of dead end products or suicide substrates (2, 5). Production of the GEM (*Pseudomonas* sp. strain B13 SN45RE) was designed to circumvent this problem by constructing a hybrid *ortho*-cleavage pathway in *Pseudomonas* sp. strain B13 which allows it to simultaneously degrade both chlorinated and methylated aromatics (5, 22). We have previously shown that by productive metabolism of the biochemically incompatible mixtures by the engineered catabolic pathway, the GEM functioned to protect the activated sludge microbial community from the ecotoxicological effects of toxic phenol mixtures (5). Using TGGE analysis of 16S rDNA sequences directly amplified from total-community DNA, in the present experiment we demonstrate the breakdown of the activated sludge community structure following shock loads of mixtures of substituted phenols and, in contrast, the maintenance of a high microbial diversity when the GEM is present. Moreover, we show that inoculation with the parental strain, *Pseudomonas* sp. strain B13, does not protect the microbial community from the ecotoxicological effects of the shock load pollutants.

MATERIALS AND METHODS

Bacterial strains. The parental strain *Pseudomonas* sp. strain B13 was isolated from sewage; it is able to degrade 3-chlorobenzoate and 4-chlorophenol (4CP) but cannot degrade mixtures of chloroaromatics and methylaromatics (4). The genetically engineered strain *Pseudomonas* sp. strain B13 SN45RE, referred to as the GEM, can simultaneously degrade mixtures of chloroaromatics and methylaromatics via a hybrid *ortho*-cleavage pathway without catabolic misrouting of substituted catechols (5, 22). The pathway is based on the modified *ortho*-cleavage pathway of *Pseudomonas* sp. strain B13. Introduction into B13 of the TOL plasmid genes from *Pseudomonas putida* mt-2 encoding toluate 1,2-dioxygenase (*xytXYZ*) and dihydroxycyclohexadiene carboxylate dehydrogenase (*xytL*), together with the positive regulator of the *xytXYZL* operon (*xytS*), expands the degradation range to include 4-chlorobenzoate and allows transformation of 4-methylbenzoate to 4-methylmuconolactone, which would accumulate as a dead-end metabolite. Recruitment of a 4-methylmuconolactone methylisomerase-encoding gene (*mmli*) from *Ralstonia eutropha* JMP134 allows transformation of 4-methylmuconolactone to 3-methylmuconolactone, which can be mineralized by B13. Mutational activation of a phenol hydroxylase of B13 further extends its degradative capacities to chlorophenols and methylphenols. All the heterologous genes have been integrated into the chromosome of B13.

Laboratory scale sewage plant. The laboratory scale sewage plant used in this study represents a model ecosystem that has been constructed and was operated by standardized procedures (Deutsche Industrie Norm) as described previously (5). It consists of two distinct units, an activated sludge unit and a clarification unit. The activated sludge unit contained 3.5 liters of activated sludge freshly obtained from the municipal sewage treatment plant in Braunschweig. The sludge was stirred at 100 rpm to prevent sedimentation. The concentration of dissolved oxygen was measured on-line, recorded, and regulated. Discontinuous aeration was triggered when the oxygen concentration dropped to a lower preset level of 2.5 mg of O₂ per liter and continued until an upper level of 3.0 mg/liter was reached. Air entered the laboratory scale sewage plant at a flow rate of 2.3 liters/min. Sterile, 50-fold-concentrated synthetic sewage (5) and a corresponding amount of dilution water were continuously added via peristaltic pumps at an overall dilution rate of 0.07/h. Activated sludge was transported by gravity flow into the 1.5-liter clarification unit, where it was allowed to settle and mixed gently by stirring slowly at 5 rpm. The settled sludge was periodically (four times per h) pumped back into the activated sludge unit. The clarified supernatant left the system via the overflow. The system was operated at room temperature.

Experiments with the laboratory scale sewage plant. Before each experiment, the laboratory scale sewage plant was filled with fresh sludge from the Braunschweig municipal sewage plant and operated undisturbed for 1 week to allow equilibration. Three different experiments were conducted. The first was a validation experiment, in which one model sewage plant was surveyed for 2 weeks to determine which changes took place in the activated sludge microbial community. The second and third sets of experiments were conducted by using the GEM and the parental strain, B13, respectively, as inoculants. In these experiments, shock loads of equimolar phenol mixtures comprised of either 4CP and 4-methylphenol (4MP) or 3-chlorophenol (3CP) and 4-methylphenol (4MP)

were added for 24 h. The concentration of each individual substituted phenol was 1 mM; i.e., the mixtures contained a total of 2×1 mM for each substituted phenol. The experimental design in the GEM experiments was such that plant 1 was given a shock (3CP and 4MP) and was inoculated with the GEM, plant 2 was given the shock but not inoculated with the GEM, and plant 3 was left untreated as a control. In the B13 experiment, plant 1 and plant 2 were both inoculated with B13 and given a shock of 3CP-4MP (plant 1) or 4CP-4MP (plant 2). Again, plant 3 was left untreated as a control. The GEM was grown in M9 minimal medium (23) supplemented with salts as described previously (33) and containing the phenol mixture (0.1 mM each) as the sole carbon and energy source. *Pseudomonas* sp. strain B13 was cultured in M9 minimal medium supplemented with salts as above and containing 4CP (0.1 mM) as the sole carbon and energy source. Inocula were grown on the respective minimal medium, harvested in the late log phase by centrifugation ($4,000 \times g$ at 4°C), washed twice in M9 buffer, resuspended in 1/10 volume of M9 buffer, and added directly to the activated sludge unit to a final density of approximately 10^7 CFU/ml. During the experiment, the inoculants maintained densities of between 10^4 and 10^6 CFU/ml, as determined by selective plate counting (5).

For DNA extraction, 1-ml samples were taken from the activated sludge unit. The on-line measurement of oxygen concentration allowed calculation of the oxygen uptake rate by the organisms in the activated sludge of the model plant as a sum parameter for their respiratory activity as described previously [5].

DNA extraction from sewage sludge. The DNA was extracted from activated sludge samples by a modified direct-lysis procedure involving physical disruption of cells (28). Sludge samples of 1 ml were subdivided into two 0.5-ml aliquots, suspended in 0.5 ml of 0.1 M sodium phosphate buffer (pH 8), supplemented with 0.13 g of lysozyme and 20 μ l proteinase K (20 mg/ml), and incubated at 37°C for 2 h. After incubation, 0.5 g of acid-washed glass beads (0.17 to 0.18 mm in diameter; Braun, Melsungen, Germany) was added and the suspensions were shaken for 4.5 min at 4°C in a bead beater (MM 2000; Retsch, Haan, Germany) at maximum speed to lyse the cells. The resulting suspensions were mixed with 100 μ l of 5 M NaCl and 62 μ l of cetyltrimethylammonium bromide (CTAB) (10%, wt/vol, in 0.7 M NaCl) and incubated at 65°C for 10 min. DNA was extracted with equal volumes of TE-equilibrated phenol (pH 7.5 to 8.0) (Roth, Karlsruhe, Germany) and centrifuged at $15,300 \times g$ for 15 min at room temperature. The aqueous phase was transferred to a new tube, and the pellet, together with the phenolic phase, was reextracted with 1 volume of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) (23). Both resulting aqueous phases were further extracted with equal volumes of phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) and chloroform-isoamylalcohol (24:1, vol/vol), precipitated with 0.7 volume of cold isopropanol and 0.1 volume of 3 M sodium acetate (pH 5.8) at -20°C overnight, and centrifuged at $>16,000 \times g$ for 30 min at 4°C. The DNA pellets were washed with 70% ethanol and dried. Finally, the dried pellets of the subsamples were resuspended in TE buffer and pooled to give a final volume of 100 μ l. The DNA preparations were applied directly in PCRs.

Primers and PCR amplification. Primers for PCR were specific for conserved bacterial 16S rDNA sequences. PCR with primers R1401 and F968GC (7) amplified a bacterial 16S rDNA fragment from positions 968 to 1401 (*Escherichia coli* numbering). A GC-rich sequence was attached to the 5' end of primer F968GC. Thus, during amplification, a GC clamp is formed, which prevents complete melting of the PCR products during subsequent separation on the denaturing gradient during TGGE. PCR amplification was performed in a total volume of 50 μ l under a layer of light mineral oil in a DNA thermocycler (TC varius V; Landgraf, Langenhagen, Germany). Each PCR mixture contained 0.5 μ l of template DNA (ca. 1.5 ng), 3 mM MgCl₂ solution, 5% (vol/vol) dimethyl sulfoxide, each deoxynucleoside at a final concentration of 0.1 mM, each primer at a final concentration of 0.1 μ M, and 0.5 U of AmpliTaq Stoffel fragment (Perkin-Elmer, Branchburg, N.J.) in Stoffel buffer (Perkin-Elmer) containing 10 mM Tris-HCl (pH 8.3) and 10 mM KCl. Amplification was performed for 35 cycles under the following conditions: after 7 min of initial denaturation at 94°C, each cycle consisted of denaturation at 94°C for 1 min, primer annealing at 54°C for 1 min, and primer extension at 72°C for 1 min, followed by a 10-min final extension step at 72°C in the last cycle. Products were visualized by electrophoresis in 0.8% (wt/vol) agarose gels after ethidium bromide staining with a TAE buffer system (23).

TGGE. The TGGE system (Qiagen, Hilden, Germany) was used as specified by the manufacturer. Aliquots (0.5 to 2.5 μ l) of PCR products were electrophoresed in gels containing 6% acrylamide, 8 M urea, and 20% formamide with a TAE buffer system (23) at a constant voltage of 100 V for 17 h, applying a thermal gradient of 39 to 52°C. Before electrophoresis, the gel was equilibrated to the temperature gradient for 30 to 45 min. A mixture of amplified 16S rDNA fragments of different soil bacteria was used as a reference pattern (11). The reference pattern consisted of amplified 16S rDNA fragments of *Erwinia carotovora* subsp. *carotovora*, *Agrobacterium tumefaciens*, *Erwinia herbicola*, *Burkholderia gladioli*, *Streptomyces aureofaciens*, *Actinomyces* sp. strain QMB-814, *Clostridium pasteurianum*, *Rhizobium leguminosarum*, *Actinosynnema mirum*, *Actinoplanes auranticolor*, and *Pseudomonas fluorescens* R2f. The gels were silver stained (21), dried and scanned (Elscrip 400; Hirschmann).

Analysis of TGGE patterns. Scanned gels were analyzed with the GelCompar software package (version 4.0; Applied Maths). A densitometric curve was calculated for each gel track. In the following normalization step, one reference sample was defined as the "standard" pattern (external reference pattern). The

five reference patterns on each TGGE gel were aligned to this external reference pattern. The banding patterns of the samples were aligned gradually according to the alignment information provided by the closest neighboring standard patterns. By aligning the bands of all references and sample tracks from every gel to the external reference pattern, it became possible to compare patterns from different gels with each other. The patterns were analyzed in two ways. (i) After assigning bands to the gel tracks, a band similarity coefficient, S_D [Dice; $S_D = (2n_{AB}) / (n_A + n_B)$], where n_A is the total number of bands in gel A, n_B is the total number of bands in gel B, and n_{AB} is the number of bands common to gel A and gel B], and the clustering algorithm of Ward (34) were used to calculate dendrograms. (ii) As a parameter for the structural diversity of the microbial community, the Shannon-Weaver index of general diversity, H (26), was calculated by using the following function:

$$H = -\sum P_i \log P_i \quad (1)$$

where P_i is the importance probability of the bands in a track. H was calculated on the basis of the bands on the gel tracks that were applied for the generation of the dendrograms by using the intensities of the bands as judged by peak heights in the densitometric curves. The importance probability, P_i , was calculated as

$$P_i = n_i / N \quad (2)$$

where n_i is the height of a peak and N is the sum of all peak heights in the densitometric curve.

RESULTS

TGGE analysis of the activated sludge microbial community. To determine the reproducibility of DNA extraction, PCR amplification, and TGGE separation of amplified fragments, an activated sludge sample was split into 10 aliquots which were extracted in parallel. The 10 subsamples generated identical TGGE banding patterns (data not shown).

The structure of the activated sludge microbial community was analyzed in three different experiments with the laboratory model sewage plant. In the validation experiment, TGGE banding patterns of an untreated plant and the untreated controls from the shock load experiments (see Fig. 3, plant 3, and Fig. 6, plant 3) were compared with each other after normalization to a standard reference pattern and are shown in Fig. 1. They revealed 10 to 18 clearly distinguishable bands per gel strip, which reflect the structure of the microbial community at this distinct point in time. To determine the information content of the banding patterns in terms of the structural diversity, they were analyzed in two ways. First, the similarities of all possible pairs of gel tracks were calculated, and then a cluster analysis of the matrix of similarity values and visualization in a dendrogram were performed (Fig. 2A). The cluster analysis revealed three major groups, which corresponded to the microbial communities in the three experiments. Thus, in each experiment a diverse microbial community with a distinct structure had established itself, which was different from the communities in the other experiments as indicated by the separate clusters. The variability of the banding patterns within these clusters indicated that the structure of the microbial communities was not static but rather dynamic.

The second method for determination of the structural diversity was the calculation of the Shannon index of diversity H from the TGGE banding pattern of a sample. H was calculated on the basis of the number and relative intensity of bands on a gel strip. By avoiding the bias of cultivation by direct extraction of DNA from the activated sludge, H can be used as a parameter which reflects the structural diversity of the whole microbial community. During all three runs of untreated plants, H showed alternating phases of higher and lower diversity (Fig. 2B), ranging between 0.92 and 1.18. These values indicate stable maintenance of a structurally diverse microbial community. Otherwise, a reduced diversity would result in less distinct bands and thus in reduced H values.

Therefore, the analysis of three different runs of the un-

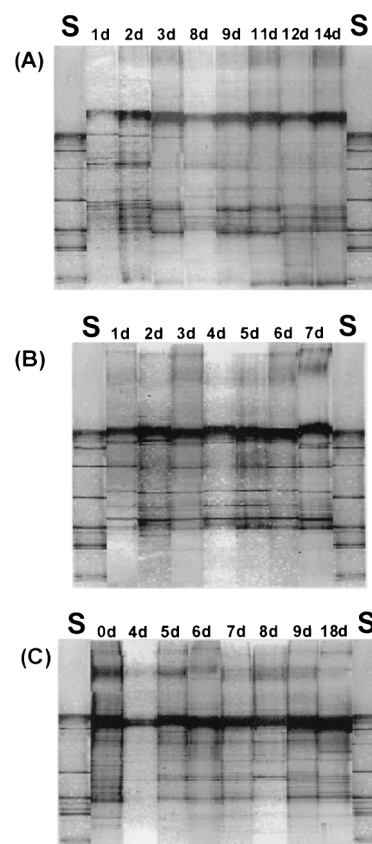


FIG. 1. Validation experiment showing TGGE banding patterns of 16S rDNA fragments amplified from activated sludge DNA of three different untreated model sewage plants after scanning of the original gel and normalization to the reference standard. The time course of the experiments is indicated above the lanes in days. (A) Validation experiment. (B and C) Untreated controls (plant 3) of shock load experiments conducted with the GEM (B) and its parental strain *Pseudomonas* sp. strain B13 (C). S, standard reference pattern.

treated control plant in the validation experiment revealed that each sample of activated sludge maintained a highly diverse and dynamic microbial community, which was stable with respect to its overall structural diversity. Communities from different samples could be differentiated from each other on the basis of their TGGE banding patterns.

Effect of phenol shock loads on the activated sludge microbial community inoculated with the GEM. The effects of a shock load of 3CP-4MP on the activated sludge microbial community in the presence of the GEM are shown in Fig. 3 to 5. Figure 3 shows the original TGGE gels. Figure 4 shows the normalized gels with the gel strips arranged in ascending time order, starting from the onset of the shock, for each plant separately. Figure 5 shows the cluster analysis of the normalized gels (Fig. 5A) and the change in the diversity index H for the GEM experiment together with the oxygen uptake rate of the microbial community (Fig. 5B).

After the shock load of the 3CP-4MP mixture was added to the model sewage plant 2, the TGGE banding patterns revealed dramatic changes in the structure of the microbial community (Fig. 4B). In plant 2, which lacked the GEM, the number of bands decreased from 11 to 4, which built a new cluster far away from the banding patterns observed before and 1 day after the shock load (Fig. 5A), indicating the collapse of the microbial community due to the shock load. One week later, the microbial community was still less diverse than be-

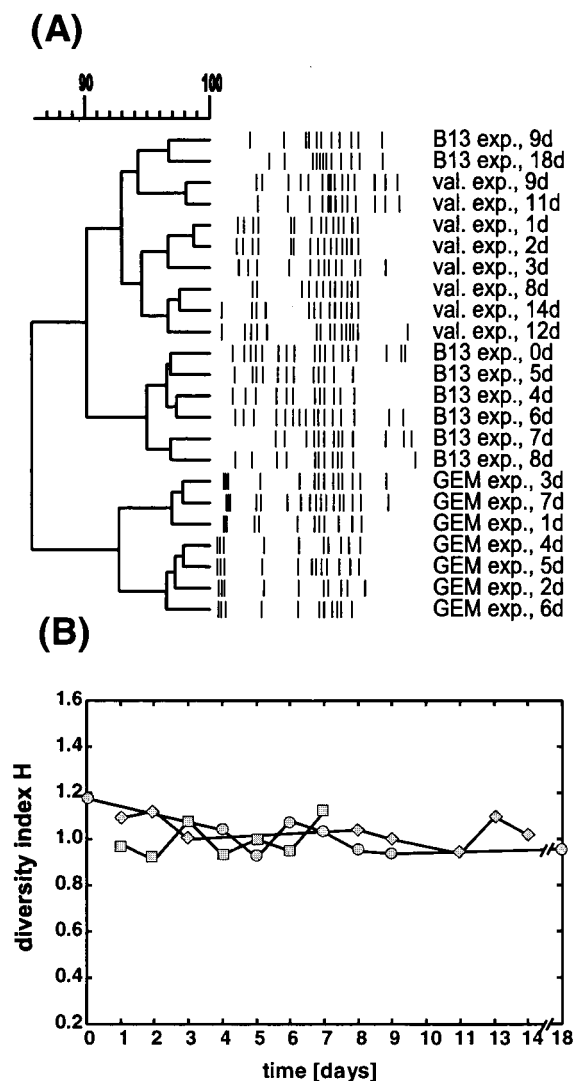


FIG. 2. Validation experiment showing analysis of the TGGE banding patterns from Fig. 1. (A) Dendrogram calculated on the basis of the Dice coefficient of similarity with the clustering algorithm of Ward. (B) Shannon index of diversity: \blacklozenge , validation experiment; \blacksquare , GEM experiment; \bullet , B13 experiment.

fore the shock. The parameter H decreased from 1.13 to 0.35 and finally to 0.22 at day 4, in contrast to the untreated control plant 3, where H remained in the range of 0.98 to 1.12 (Fig. 5B). Subsequently, in plant 2 the value of H increased slightly to 0.49 but remained clearly below the control ($H = 1.12$), although the phenol feed had been terminated after 24 h. The oxygen uptake rate Q_{O_2} , as a sum parameter for microbial activity, decreased rapidly from values in the range of 0.40 to 0.45 to $<0.1 \text{ mg of } O_2 \cdot \text{liter}^{-1} \cdot \text{min}^{-1}$ within 5 to 8 h (Fig. 5B). The oxygen uptake rate indicated a slight increase in microbial activity after termination of the phenol stress, but it remained clearly below the activity level of the community before the shock load. In contrast to the oxygen uptake rate, which decreased within 5 to 8 h after the shock, the effect of the phenol mixtures on the microbial community structure were detectable by TGGE only after 2 days.

In plant 1, the activated sludge community was protected against the toxic effects of the phenol mixture because the GEM degraded the biochemically incompatible mixture, largely preventing the misrouting of chlorophenols into unproduc-

tive *meta*-cleavage routes and hence preventing the formation of toxic dead-end metabolites (5). The oxygen uptake rates were slightly reduced but stabilized at $0.3 \text{ mg} \cdot \text{l}^{-1} \cdot \text{min}^{-1}$, indicating continued microbial respiration during and after the

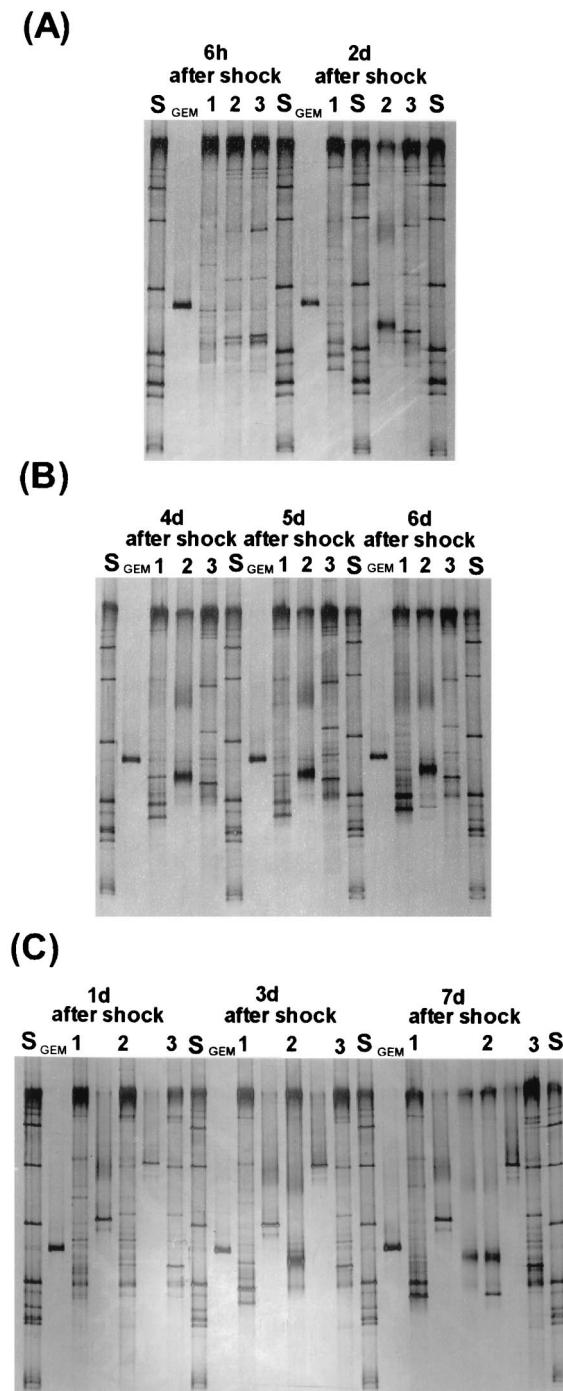


FIG. 3. GEM experiment showing original TGGE gels of amplified 16S rDNA fragments from activated sludge microbial communities given shock loads of 3CP-4MP (1 mM each) and simultaneously inoculated with GEM (plant 1) or lacking the GEM (plant 2). Plant 3 was an untreated control. The sampling time is indicated in days after the start of the phenol shock load. Lanes: S, standard reference pattern; GEM, pure culture of the GEM; 1, shock-loaded plant inoculated with the GEM; 2, shock-loaded plant lacking the GEM; 3, untreated control plant. Panels A, B, and C show individual TGGE gels.

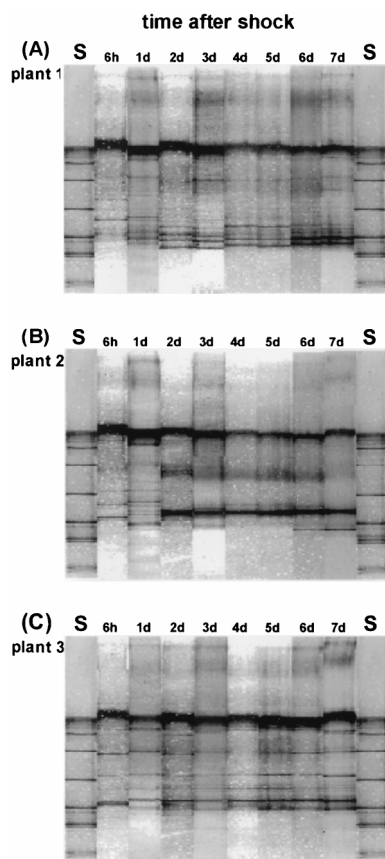


FIG. 4. GEM experiment showing normalization of the TGGE gels from Fig. 3 to the reference standard. Gel strips are sorted in ascending time order for each plant separately. The sampling time is indicated above the gel strips. S, Standard reference pattern. (A) Plant 1, shock-loaded plant 1 inoculated with the GEM; (B) plant 2, shock-loaded plant 2 lacking the GEM; (C) plant 3, untreated control.

shock loading (Fig. 5B). No significant changes were detectable in the banding patterns of plant 1 before and after the shock (Fig. 4A and 5A). The values of H were reduced somewhat from 1.03 to 0.93 on day 2 and finally to 0.82 on day 4 but recovered to the level of the untreated control on day 5 (Fig. 5B). These results are consistent with our previous results (5) and provide additional evidence for the bioprotection of microbial communities from toxic phenol mixtures by the GEM *Pseudomonas* sp. strain B13 SN45RE.

Effect of phenol shock loads on the activated sludge microbial community inoculated with the parental strain *Pseudomonas* sp. strain B13. Figures 6 and 7 illustrate the analysis of the B13 experiment, starting with the original gels (Fig. 6) and then showing the cluster analysis of the banding patterns (Fig. 7A) and the diversity value, H , together with the oxygen uptake rate of the microbial communities (Fig. 7B). Initially, the community structure was identical in the three plants filled with freshly obtained sewage, as shown by the original TGGE gel (Fig. 6A) and the cluster analysis (Fig. 7A). During the adaptation of the activated sludge to the laboratory model sewage plant conditions prior to application of the shock, changes in community structure occurred. In all three plants, the Shannon index of diversity, H , decreased slightly from 1.01–1.20 to 1.05–0.92. The changes in the community structure were similar in the three plants operated in parallel, as evidenced by the results of the cluster analysis (Fig. 7A), and thus show the similarity of the three plants. In the shock load experiment with

the parental strain *Pseudomonas* sp. strain B13, the inoculated plants were given shock loads of 4CP-4MP or 3CP-4MP mixtures for 24 h, as described in Materials and Methods. Strain B13, which is capable of metabolizing 4CP but not 3CP, failed to protect the microbial community. The shock loads led to a dramatic decrease of the oxygen uptake rate (Fig. 7B), indicating the breakdown of metabolic activity in the activated sludge microbial community. The effect of the phenol shock on the structure of the microbial community was again visible a few days later in the TGGE analysis. On days 3 and 4 after the phenol mixtures were added to plant 1 and plant 2 (both inoculated with B13), the breakdown of the microbial commu-

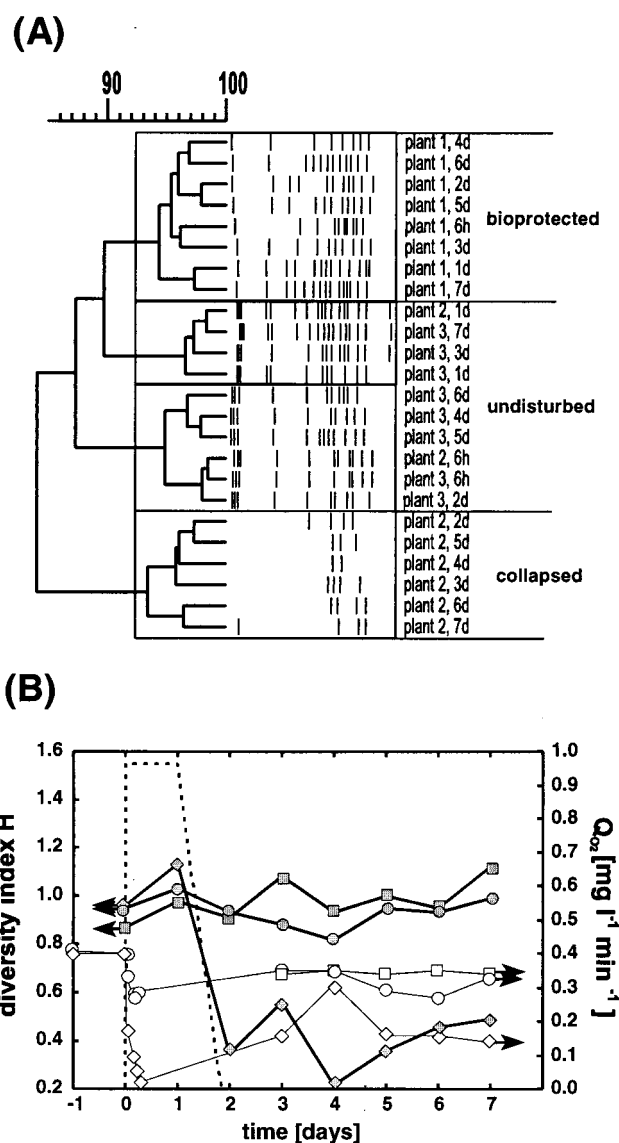


FIG. 5. GEM experiment showing analysis of the TGGE banding patterns from Fig. 4. (A) Dendrogram calculated on the basis of the Dice coefficient of similarity with the clustering algorithm of Ward. The terms "bioprotected," "undisturbed," and "collapsed" were assigned to the clusters to describe the status of the microbial communities during the shock load experiment. (B) Shannon index of diversity, H (shaded symbols), and oxygen uptake rate, Q_{O_2} (open symbols), of the activated sludge microbial communities during the GEM experiment: ●, ○, shock-loaded plant 1, inoculated with the GEM; ◆, ◇, shock-loaded plant 2, lacking the GEM; ■, □, plant 3, untreated control. The duration of the phenol shock load is indicated by the dotted line.

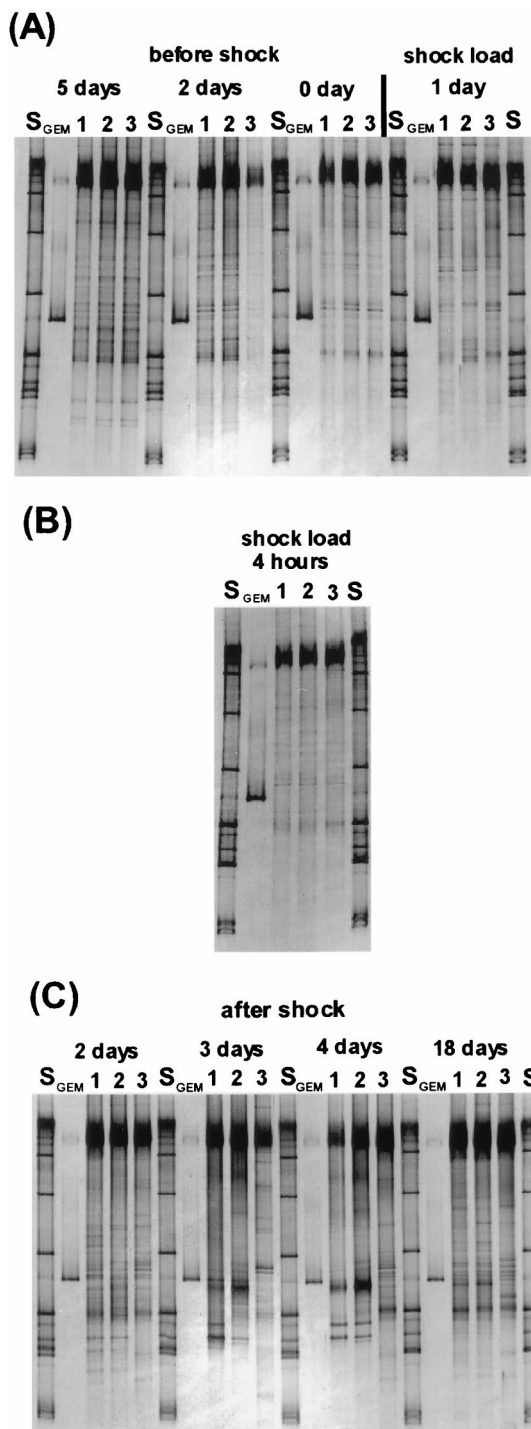


FIG. 6. B13 experiment showing original TGGE gels of amplified 16S rDNA fragments from activated sludge microbial communities given shock loads of substituted phenols (1 mM each individual phenol) and simultaneously inoculated with *Pseudomonas* sp. strain B13. The sampling time is indicated in hours or days relative to the start of the phenol shock. Lanes: S, standard reference pattern; GEM, pure culture of the GEM; 1, plant 1, inoculated with B13 and given a shock load of 3CP-4MP; 2, plant 2, inoculated with B13 and given a shock load of 4CP-4MP; 3, plant 3, untreated control plant.

nities was clearly visible in the TGGE banding patterns (Fig. 6C). Cluster analysis revealed three separate clusters, which corresponded to the undisturbed activated sludge communities before the shock, the collapsed community, and an intermedi-

ate cluster with some elements similar to the collapsed group but moving back toward reestablishment of diversity (Fig. 7A). The Shannon index of diversity allowed the detection of slight differences in the responses of the communities to the 3CP-4MP shock in plant 1 compared to the 4CP-4MP shock in plant 2 (Fig. 7B). For the 3CP-4MP mixture in plant 1, H decreased from 1.22 to 0.46, whereas in plant 2, containing the 4CP-4MP mixture, the decrease from 1.03 to 0.70 was less pronounced. This difference might be due to the higher toxicity of the 3CP-4MP mixture for the microbial community (2, 5) and to the fact that 4CP is being degraded by B13 while 3CP is not (2, 4).

At 17 days after termination of the phenol shock load, H had recovered to its original value (0.93 to 0.89) in all three plants.

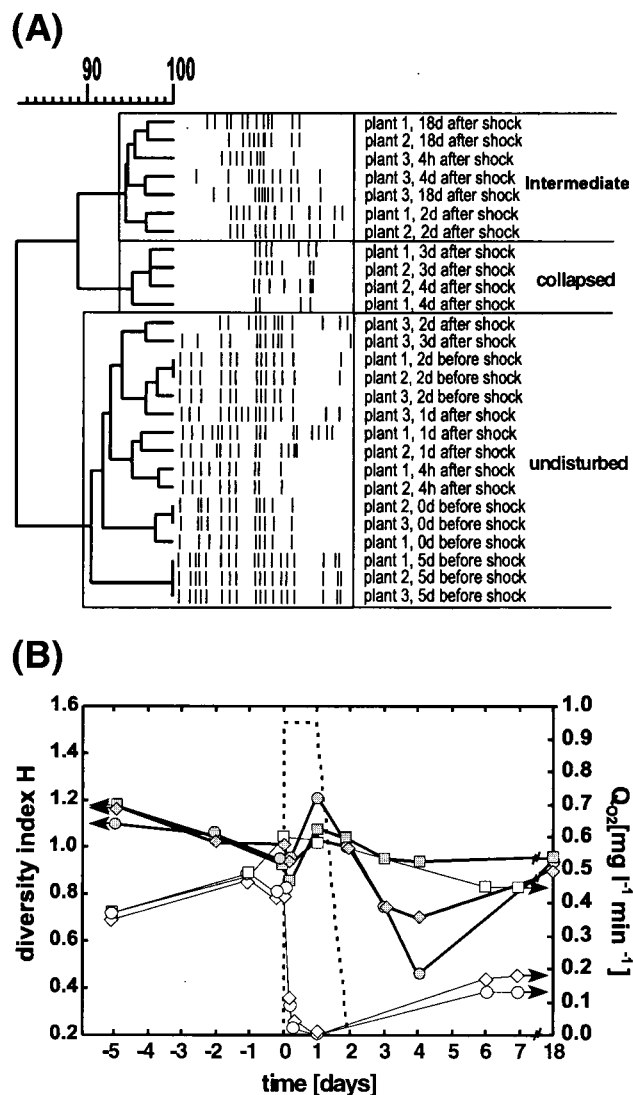


FIG. 7. B13 experiment showing analysis of TGGE banding patterns from Fig. 6. (A) Dendrogram calculated on the basis of the Dice coefficient of similarity with the clustering algorithm of Ward. The terms "intermediate," "collapsed," and "undisturbed" were assigned to the clusters to describe the status of the microbial communities during the shock load experiment. (B) Shannon index of diversity, H (shaded symbols), and oxygen uptake rate, Q_{O_2} (open symbols), of the activated sludge microbial community during the B13 experiment: ●, ○, plant 1, inoculated with B13 and amended with the 3CP-4MP mixture; ◆, ◇, plant 2, inoculated with B13 and amended with the 4CP-4MP mixture; ■, □, plant 3, untreated control. The duration of the phenol shock load is indicated by the dotted line.

Small differences between the banding patterns of plants 1, 2, and 3 were visible, but they all clustered closely. Therefore, the microbial communities in the model sewage plant had recovered from the ecotoxicological effects of the phenol mixtures and reestablished themselves to form a different but again highly diverse microbial community.

DISCUSSION

Community diversity. Community diversity is a key concept in ecology, and its quantification is fundamental for analyzing phenomena such as succession, colonization, and, as in the study reported here, response to disturbances. The Shannon index of diversity was introduced into ecology by Shannon and Weaver (26) and is still widely used in macroecology (see e.g., reference 14). However, it is difficult to apply this index to microbial communities, since the number and relative abundances of species cannot be determined comprehensively. Therefore, the diversity of microbial communities has been estimated indirectly from the heterogeneity of total-community DNA (30), from the restriction fragment length polymorphism patterns of amplified ribosomal sequences (15), or from biochemical and genetic fingerprint techniques applied to cultivated bacteria (see, e.g., references 12 and 32). To circumvent the problem of defining a microbial species, Watve and Gangal (35) suggested a dissimilarity index based on the taxonomic distances between "biotypes," which, however, fails to consider their relative abundances and requires cultivation or 16S rDNA clone libraries. In this study, we applied the Shannon index of diversity to ribosomal sequences amplified directly from community DNA and separated on a denaturing gel according to sequence heterogeneity. Using both the number and relative intensities of rDNA bands on the TGGE gel, we calculated the diversity index, H , which reflects the diversity of abundant ribosomal gene sequence types within the community without the need for cultivation.

TGGE analysis of rDNA. Separation of nucleic acids on a denaturing gel has a very high sensitivity. Under optimized conditions, one point mutation in a 1,000-bp fragment may be detected (27). However, the number and intensity of bands do not equal the number and abundance of species within the microbial community, due to features of 16S rDNA-based phylogeny on the one hand and to bias inherent to PCR amplification from complex template mixtures on the other. One organism may produce more than one TGGE band because of multiple, heterogeneous rRNA operons (3, 17, 19). Conversely, for some phylogenetic groups of bacteria, 16S rDNA sequences do not allow discrimination between species, so that one TGGE/DGGE band may represent several species with identical rDNA sequences (31). Banding patterns are subject to the bias inherent to PCR-based techniques, e.g., selectivity of DNA extraction, potential preferential amplification, and chimera formation (36). In a complex mixture of target rDNAs, less abundant sequences are not amplified sufficiently to be visualized as bands. Therefore, the banding pattern reflects the most abundant rDNA types of the microbial community. The diversity index calculated from the TGGE banding patterns of amplified 16S rDNA sequences is therefore a relative term. It is independent of cultivation and requires no information about the species composition of the community analyzed. Here we have shown that the diversity index is applicable to complex microbial communities and is especially well suited for comparing large sets of samples from the same habitat.

For the banding patterns analyzed here, a maximum of 18 distinct bands on the TGGE were found, which, at equal in-

tensity per band, corresponds to a diversity index, H , of 1.24. When there is an increasingly uneven distribution of bands, e.g., some dominant and few less intense bands, H decreases. If the pattern were composed of one dominant band (90% of total intensity) and 17 bands which together make up the remaining 10%, H would be 0.27. This calculation shows that minor bands do not significantly influence the value of H .

The TGGE banding pattern of amplified rDNA sequences from activated sludge was reproducibly generated, and thus a meaningful comparison of banding patterns between samples was possible. By using the Shannon index of diversity in combination with the cluster analysis of the TGGE banding patterns based on the similarity coefficient, we were able to monitor a whole range of community responses. First, we documented clearly the stability of the microbial community within the laboratory scale sewage plant during the experimental period and the identity of triplicate model systems made up from the same sewage batch. Moreover, subtle shifts in community structure during adaptation to laboratory conditions, which are commonly observed if comparatively small natural samples are maintained in the laboratory in microcosms ("bottle effect"), could be demonstrated. The microbial community was shown to recover within 18 days after the shock; recovery involved restoration of the original diversity but a different banding pattern from before the shock load. Finally, complete breakdown of the community structure after phenol shock loads and the bioprotective effect of a highly specific inoculant, namely, the GEM *Pseudomonas* sp. strain B13 SN45RE, could clearly be demonstrated. Thus, quantitative TGGE analysis proved to be a powerful tool to monitor a whole range of changes in community structure, allowing the high sample throughput which is required for investigating ecological questions.

Structural and functional community responses to phenol shocks. The activated sludge microbial community responded rapidly to the pollutant shock by reducing its oxygen uptake rate to $<0.1 \text{ mg of O}_2 \cdot \text{liter}^{-1} \cdot \text{min}^{-1}$ within less than 5 to 8 h after the beginning of the shock. The density of culturable bacteria decreased by 3 orders of magnitude (5). However, the breakdown in microbial community structure became visible on the banding patterns of amplified rDNA sequences only 2 to 4 days after application of the shock load of phenols. The DNA of the bacteria that had been killed by the phenol shock obviously persisted in the sewage model plant. It has to remain open whether the DNA was released from killed bacterial cells and was protected from DNase attack (6, 18) because of protein denaturation due to the phenols (25) or if the DNA remained within the dead cells and thus was protected from degradation by DNase enzymes (13). After the phenols had disappeared completely from the plant and the respiratory activity of the microorganisms had started to recover, the DNA of the killed bacteria was degraded and the change in community structure became visible on the TGGE gels.

Bioprotection. We amended the complex, highly active and dynamic activated sludge microbial community with a microorganism carrying a catabolic trait lacking in natural microbial communities. We demonstrated, by comparison with the parental strain, that the pathway for simultaneous degradation of mixtures of chlorinated and methylated phenols constructed by genetic engineering was essential for bioprotection by the GEM. We were thus able to protect the sewage sludge system from the ecotoxicological effects of a pollutant mixture and ensure maintenance of the waste treatment process. By analyzing microbial community structure by a culture-independent approach, namely, TGGE analysis of amplified rDNA sequences, we were able to demonstrate the maintenance of a

high microbial diversity in the bioprotected plant, confirming our previous results (5). Bioprotection by inoculation might be a useful approach to eliminate a range of hazards from sewage plants. TGGE analysis of community structure is a powerful tool to analyze the efficiency of potential inoculants in protecting microbial communities and their activities.

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